The Physiology of Salivary Secretion

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Abstract.

Saliva in the mouth is a biofluid produced mainly by three pairs of major salivary glands, the submandibular, parotid and sublingual glands, along with secretions from many minor submucosal salivary glands. Salivary gland secretion is a nerve mediated reflex and the volume of saliva secreted is dependent on the intensity and type of taste, chemosensory, masticatory or tactile stimulation. Long periods of low (resting or unstimulated) flow are broken by short periods of high flow stimulated by taste and mastication. The nerve-mediated salivary reflex is modulated by nerve signals from other centres in the central nervous system, most obvious as hyposalivation at times of anxiety. An example of the other neurohormonal influences on the salivary reflex is the circadian rhythm seen in salivary flow and ionic composition. Cholinergic parasympathetic and adrenergic sympathetic autonomic nerves evoke salivary secretion, signaling through muscarinic M3 and adrenoceptors on salivary acinar cells leading to secretion of fluid and salivary proteins. Saliva gland acinar cells are chloride and sodium secreting and the isotonic fluid produced is rendered hypotonic by salivary gland duct cells as it flows to the mouth. The major proteins present in saliva are secreted by salivary glands creating viscoelasticity and enabling the coating of oral surfaces with saliva. Salivary films are essential for maintaining oral health and regulating the oral microbiome. Saliva in the mouth contains a range of validated and potential disease biomarkers derived from epithelial cells, neutrophils, the microbiome, gingival crevicular fluid and serum. For example, cortisol levels are used in the assessment of stress, matrix metalloproteinases (MMP8 & 9) appear to be promising markers of caries and periodontal disease and a panel of mRNA and proteins have been proposed as a marker of oral squamous cell carcinoma. Understanding the mechanisms by which components enter saliva is an important aspect of validating their use as biomarkers of health and disease.

Introduction.

Saliva performs a number of important functions that are essential for the maintenance of oral health. Most of these functions depend upon interaction of saliva with a oral surfaces of varying texture and polarity; soft epithelial tissue surfaces with differing degrees of keratinization and roughness along with the tooth surfaces which are hard and composed of tooth mineral. Saliva clears substances from the mouth, buffers pH, maintains tooth mineralization, facilitates wound healing, neutralizes some harmful dietary components, influences the oral microbiome, protects, lubricates and hydrates oral mucosal surfaces. The properties and effectiveness of saliva are largely determined by secretions from the major and minor salivary glands. Saliva is an accessible biofluid that contains components derived from the mucosal surfaces, gingival crevices and tooth surfaces of the mouth. Saliva also contains microorganisms that colonize the mouth and other exogenous substances and so can potentially give an insight into the relationship of the host with the environment. These features make saliva a complex fluid. It is therefore important to understand how saliva is formed so that we can make informed interpretations of changed salivary composition linked with physiology or disease.

Salivary gland anatomy and structure

Salivary glands are exocrine glands and secrete onto a mucosal surface. During embryonic development major salivary glands form as initial proliferating epithelial buds that form from the oral epithelium and grow into the underlying mesenchyme. A tree-like ductal structure develops through a process of branching morphogenesis and canalization. The development process requires a controlled exchange of molecular signals between epithelial cells and mesenchymal cells (60). The ductal structure of the major adult salivary glands is well demonstrated by sialography, an imaging technique whereby x-ray contrast medium is injected into the opening of the main excretory duct of the gland on the oral epithelium; Stenson's (parotid) duct or Wharton's (submandibular) duct (Figure 1).

Figure 1 near here.

At the ends of fine branches of the major salivary gland ductal tree are glandular secretory endpieces referred to as acini (grape-like), which are collections of saliva secreting epithelial cells. The mechanisms by which acinar cells secrete saliva are discussed later but the histological appearance of acinar cells is determined by the types of secretory proteins synthesized by the cells and stored in large granules, which can fill the cytoplasm. The content of the storage granules is an indicator of the types of saliva produced which can be broadly divided into mucin containing and non-mucin containing salivas. Mucins are the main components of mucous, a protective layer found on most mucosal surfaces in the body, and salivas containing greater amounts of mucin tend to be viscoelastic; an important characteristic for retention of saliva on oral mucosal surfaces and maintenance of lubrication and hydration of the surfaces (94). Parotid gland acinar cells produce watery saliva with little or no mucin and characteristically stain strongly with the routinely used histological dyes haematoxylin and eosin (H&E; Figure 2a). The submandibular gland contains a mixed population of acinar cells, some of which are mucin producing and pale-stained with H&E. Most of the acinar cells in sublingual glands are mucin producing and consequently the saliva secreted tends to be viscoelastic. Some acinar cells in these glands contain less mucin, possibly because it is not fully formed within the storage granules of cells, and have a 'serous' appearance with H&E staining. Most of the minor salivary glands in the oral submucosae are mucin producing and the acinar cells stain similarly to those of the sublingual gland (Figure 2b). The histological dye combination of Periodic Acid Schiff reagent and Alcian Blue can be used to stain mucins and demonstrates how fewer acini of submandibular gland are mucin producing compared to the sublingual gland (Figure 2c & d) (86).

Figure 2 near here.

Following secretion saliva enters the ductal system moving through the smallest (intercalated) ducts, through striated ducts, interlobular collecting ducts and finally the single main excretory of the gland.

Myoepithelial cells surround acini and intercalated ducts and provide contractile support to these structures (Figure 2e). The process of salivary secretion is dependent upon a rich supply of arterioles surrounding ducts and acini and a rich autonomic innervation with parasympathetic and sympathetic nerves supplying acinar and ductal cells (28). Minor salivary glands lie just beneath the oral mucosal surface in the submucosa and thus have a short ductal system with intercalated like duct cells but fewer recognizable striated duct cells. The interlobular collecting ducts tend to open directly onto the mucosal surface (Figure 2b). Minor salivary glands are predominantly mucous secreting glands as can be seen by everting the lower lip and observing the beads of mucin rich saliva that appear over ductal openings on the oral mucosa. Serous minor salivary glands (of von Ebner) are present in association with the circumvallate papillae of the tongue.

The salivary reflex

Secretion from the major salivary glands is evoked by interaction of tastants with different receptors on taste buds located predominantly in the epithelium on the dorsum of the tongue and following activation of mechanoreceptors in the periodontal ligament and mucosae (41). Minor salivary glands may also increase secretion in response to taste stimulation (83) but perhaps movement and tactile stimulation of the mucosa play a more important role in labial and palatine minor glands (14, 91). The submandibular and sublingual glands but not the parotid gland increase secretion in response to different smells associated with food (50). The sensation of cold in the mouth can evoke a flow of saliva (Lee et al., 2006) and can increase salivation in response to liquid gustatory stimulation. Temperature, pungent substances such as capsaicin and hydroxyl-alpha-sanshool and cooling agents such as menthol activate Transient Receptor Potential (TRP) channels and a range of these channels are expressed on trigeminal nerve endings, taste receptors and oral keratinocytes (92) and some have been shown to evoke salivary secretion (24, 53) . Taste, mechanical or pungent signals generate signals in afferent fibres of the facial (CN VII), glossopharyngeal (CN IX) and trigeminal (CN V) nerves. The nucleus of the solitary tract is innervated by the CNVII and CNIX and sends interneurons to the salivary centres, respectively the superior and inferior salivary nuclei in the medulla

oblongata. Efferent nerve fibres from the salivary nuclei conduct efferent signals via the chorda lingual nerve to the submandibular ganglion and thence to the submandibular and sublingual glands. The parotid gland is supplied by efferent fibres in the glossopharyngeal (tympanic branch) nerve to the otic ganglion and post-ganglionic fibres in auriculo-temporal nerve (Figure 3). Minor salivary glands are supplied by parasympathetic nerve fibres in the buccal branch of the mandibular nerve, the lingual nerve and the palatine nerve.

Figure 3 - near here

The salivary reflex is profoundly influenced by central nerves from other nuclei in the brain supplying the salivary nuclei in the medulla oblongata (46). This central neural activity appears to contribute towards the resting rate of salivary secretion into the mouth since salivary flow rates are lower during sleep and virtually absent during anaesthesia. Both excitatory (gamma aminobutyric acid containing) and inhibitory (glycine containing) nerves synapse with the salivary centres (15). Suppression of impulse traffic from the salivary nuclei to salivary glands leading to reduced salivation and dry mouth is most obviously demonstrated during fear and anxiety and like other autonomic regulation, involves a complex interaction with higher (limbic and cortical) centres in the brain. Different sensory modalities, including auditory, visual and somatosensory, are associated with fear and may potentially impact on salivary secretion through pathways in the amygdala, the hypothalamus and the brainstem. It is generally assumed that the thought of food causes salivation and mouth-watering (84). However, there is no evidence of a conditioned salivary reflex in man, as observed by Pavlov in his experiments on dogs. Mouth-watering experiences may result from smell-evoked submandibular/ sublingual salivary flow (50) or may possibly be caused by muscular compression of the main salivary excretory ducts and expulsion of the saliva.

Salivary gland cells are intimately associated with the autonomic nervous system (28). Parasympathetic and sympathetic and nerves run together with Schwann cells

to the target cells in salivary glands (30). Parasympathetic and sympathetic nerves are in contact with many cell types in salivary glands including acinar, ductal, myoepithelial cells and blood vessels. The extent of innervation of salivary glands by sympathetic nerves varies greatly; the parotid and submandibular glands of rat, mouse and man receive extensive sympathetic innervations whilst mucous secreting glands such as the rat and human sublingual and the human minor salivary glands receive a sparse adrenergic innervation which appears to be directed to the vasculature rather than the parenchyma (29, 78). In addition to the main neurotranmitters acetylcholine and noradrenaline there are a range of neuropeptides, including substance P and vasoactive intestinal peptide (VIP), within nerves in salivary glands (25). Neuropeptide containing nerves supply blood vessels and parenchymal cells and show distinct innervations patterns, for example VIP containing nerves are more numerous around the mucous acinar cells in the human submandibular gland (48). Some neuropeptides are also found in sensory nerve fibres around ducts and blood vessels within the salivary glands (24).

The acute control of salivary secretion and blood flow was demonstrated using animal models under anaesthesia and has been reviewed previously (73, 74). Assay of salivary protein concentration reveals that sympathetic nerve stimulation evokes a protein-rich secretion whilst parasympathetic stimulation evokes a larger volume of saliva. Dual nerve stimulation experiments have demonstrated that the individual actions of the nerves, particularly protein secretion evoked by the sympathetic nerve, are augmented in rat parotid (7) and submandibular glands (5, 18) . Such dual stimulation experiments are thought to better reflect the events leading to reflex secretion of saliva, since it is expected that both parasympathetic and sympathetic impulses are acting on secretory cells simultaneously. An intact parasympathetic innervation is crucial to evoking a normal flow of saliva. Studies in man and the rat have demonstrated that sympathetic impulses make a contribution to the amount of protein secreted under reflex taste stimulation (57). Although adrenergic signalling from sympathetic nerves leads to an augmentation of protein secretion by parotid and submandibular glands mucin secretion from mucous glands such as the human sublingual and minor glands is dependent upon parasympathetic stimulation and peptidergic stimulation(20).

Coupling of the nerve mediated reflex to glandular secretion of salivary fluid.

The dependence of salivary secretion on acetylcholine signaling is demonstrated in cases of poisoning with the berry of Deadly Nightshade (*Atropa belladonna*), which contains high concentrations of the alkaloid atropine, an antagonist of cholinergic muscarinic receptors; poisoning is characterized by a very dry mouth in addition to the ventricular fibrillation, dizziness and other effects of muscarinic blockade. Acetylcholine released from parasympathetic nerves acts on muscarinic receptors on salivary acinar cells by acetylcholine released from parasympathetic nerves (74); mainly m3 muscarinic receptors with some contribution from m1 muscarinic receptors (33, 62). Salivary secretion is largely dependent upon the activation of Acinar cell activation of fluid transport is achieved through intracellular formation of inositol triphosphate (IP3) and elevated calcium concentration and activation of ion transporting proteins (27). Cytoplasmic calcium levels are tightly controlled by rapid removal of calcium through the actions of plasma membrane and endoplasmic reticulum calcium pumps (PMCA and SERCA). Sustained salivary secretion requires influx of extracellular calcium across the plasma membrane of acinar cells referred to as store operated calcium entry (SOCE) (4, 58). Other receptors (alpha1-adrenoceptor; substance P Neurokinin 1 receptor; P2Y receptor; P2X receptors) utilize intracellular calcium signalling mechanisms but may make comparatively minor contributions to salivary fluid secretion under physiological conditions .

Figure 4 – near here

Coupling of the nerve mediated reflex to glandular secretion of salivary protein.

Exocytosis of protein storage granules by salivary acinar cells is principally activated by noradrenaline release from sympathetic nerve endings binding to β_1-adrenoceptors and increases in G-protein coupled adenylate cyclase activity with the generation of increased levels of intracellular cAMP (11). Signalling from parasympathetic nerves can also give rise to substantial salivary protein secretion via

release of vasointestinal polypeptide (56) which also acts through increases in intracellular cAMP. However, cholinergic stimuli alone can give rise to the release of protein by a coupling mechanism independent of cAMP, involving elevated intracellular calcium and activation of protein kinase C (61).

Simultaneous activation of sympathetic and parasympathetic nerve supplies as occurs during reflex secretion, leads to 'augmented' secretion of amylase and other salivary proteins (6) and appears to reflect a 'cross-talk' between the intracellular calcium and cAMP secretory signalling pathways (13, 87). Denervation experiments in animal models have also revealed how the branches of the autonomic nervous interact during coupling of nerve stimuli to secretion (19, 71).

Mechanisms of glandular salt and water secretion by acinar cells.

The directional movement of salivary fluid and protein into acinar lumena of salivary glands and to the mouth is dependent upon salivary acinar cell polarity, that is, the apical pole of the cell has a cell membrane which contains different ion transport proteins compared to the opposite (basolateral) pole. The cell polarity is created by close interaction between adjacent cells with the formation of tight junctions and is maintained by interaction of the basal aspect of cells with basal laminae and the connective tissue matrix of the gland. Tight junctions are protein complexes formed principally by interaction of transmembrane proteins of adjacent cells. The tight junctions of acinar cells allow the movement of some ions, water and small molecules and may therefore be considered to be 'leaky' tight junctions. In the ductal system of salivary glands the ductal epithelial cells are similarly polarized but in this case the tight junctions are watertight indicative of a greater number of tight junctional contacts between cells; similar differences in the leakiness of tight junctions are seen in different parts of the kidney tubular system (9).

Figure 5 near here

Acinar cells secrete salivary fluid and there is a minimal contribution to overall volume of secretion by the ductal system through which saliva passes to the mouth. Salivary acinar cells are salt secreting and it is the movement of salt across the acinar epithelium from tissue fluid into acinar lumena that leads to water movement and formation of salivary fluid. Movement of salt across acinar cells is possible because of the activity of the sodium/ potassium ATPase (sodium pump) located in the basolateral membrane of acinar cells which maintains a low intracellular sodium concentration relative to the extracellular tissue fluid (26). This difference in sodium concentration, the sodium gradient, provides the impetus for movement of ions (principally sodium and chloride). Inhibition of sodium pump activity with the alkaloid ouabain inhibits salivary secretion (16).

Salivary secretion is also dependent upon a chloride channel (TMEM16A;(77)) in the apical membrane of acinar cells (Figure 5A); when intracellular calcium is increased during stimulation (see above) the chloride channel opens and chloride is released into the acinar lumen. Sodium follows the movement of chloride into the acinar lumin by a paracellular (passing between cells) route. The accumulation of salt in the acinar lumin leads to movement of water by osmosis, most likely by both paracellular and transcellular routes. Water movement through the acinar cell is possible because of a water channel (aquaporin 5) present in apical membranes of acinar cells (Figure 5B) (38) (55). Water therefore is drawn into the acinar lumen and ductal system either by flow through aquaporin channels or around cells and through the tight junctions. The continued movement of salivary fluid is possible because of a sodium, potassium and chloride co-transporter protein (NKCC1; (58)) on the basolateral membrane of acinar cells that allows entry of chloride (coupled with movement of sodium along its concentration gradient) into the cell to replace chloride lost across the apical membrane into the acinar lumen (Figure 5C).

Salt absorption by glandular ductal cells.

Saliva entering the mouth from major salivary salivary glands is hypotonic enabling the tasting of salt in food. Saliva secreted by acinar cells is isotonic and as it flows through the ductal system of the major salivary glands salt is removed, principally by

striated duct cells, and saliva is rendered hypotonic. The degree of hypotonicity is dependent upon salivary flow rate; consequently stimulated saliva secreted at an increased flow rate has a higher salt concentration (90, 95). The removal of sodium and chloride by ductal cells is dependent upon creation of a transmembrane gradient for sodium by the sodium potassium ATPase (sodium pump) located on the basolateral membrane. In fact striated duct cells are particularly enriched in this enzyme and with the abundance of basolaterally located ATP generating mitochondria, are well equipped to transport large amounts of salt out of the cell and into the glandular interstitium (79, 93). Sodium ions are absorbed by ductal cells from the ductal lumen through a sodium channel in the apical membrane and as sodium enters the cell it is removed across the basolateral membrane by the sodium pump. Membrane ion transporting proteins also remove chloride from saliva in the ducal lumen, across the ductal cell and into the interstitium (Figure 5)(79).

Bicarbonate is an important component of saliva since it plays a major role in buffering salivary pH near neutrality and preventing dissolution of tooth mineral, which increases in the presence of acid (see later). Salivary acinar cells can secrete bicarbonate but it appears that ductal cells also play a major role in bicarbonate secretion into saliva. Since the bicarbonate concentration of stimulated saliva is many times higher than unstimulated saliva ductal bicarbonate secretion is most likely stimulated by autonomic nerves (49, 76). Other ions transported by salivary gland cells including calcium, phosphate, thiocyanate, iodide and nitrate fulfill important functions (see later). Calcium appears to enter saliva predominantly by being packaged in protein storage granules and released during protein exocytosis (see below). The calcium concentration of glandular saliva does not vary greatly under different stimulation conditions. Phosphate secretion by salivary glands is less well understood but there are phosphate transporting proteins in membranes of salivary gland cells. Thiocyanate, iodide and nitrate are all actively transported into saliva from the circulation/ gland tissue fluid. There are membrane transporting proteins in salivary duct cells (called the sodium iodide symport and sialin) which transport these ions into saliva (76).

Protein secretion by salivary gland cells.

Most salivary gland protein secretion is due to exocytosis of protein storage granules in acinar cells. When cells are stimulated via autonomic nerves storage granules fuse with the apical membrane of acinar cells and the content of protein is released into saliva (3, 81). The packaging of proteins into storage granules at high concentrations requires accumulation of calcium ions to shield the high density of negative charges , particularly in the case of granules storing mucins which are large, highly glycosylated, negatively charged proteins (3).

Some proteins are secreted into saliva by other mechanisms referred to as vesicular transport and under experimental conditions it is possible to adjust conditions of autonomic stimulation so that similar quantities of protein are secreted as seen with storage granule secretion but without obvious loss of storage granules from acinar cells (7). Such vesicular transport also occurs in the absence of fluid secretion and can lead to accumulation of secretory proteins in the ductal system of salivary glands (31, 32, 75). Studies in vitro have demonstrated rapid secretion of newly synthesised radiolabelled secretory proteins via a vesicular pathway that can be upregulated by low doses of autonomimetics (45). The composition of proteins secreted by storage granules and vesicles differs and the mechanisms enabling selective sequestration of different proteins are still being studied in a variety of exocrine cells including salivary acinar cells (37).

Secretory Immunogobulin A (a dimeric form of IgA with bound J chain) is secreted by plasma cells present in the interstitium of salivary glands and enters saliva as SIgA. The mechanism of salivary secretion requires IgA to bind to a receptor (epithelial polymeric immunoglobulin receptor; pIgR) on the basolateral membrane of salivary acinar and ductal cells and the receptor transports the IgA across the cell and into the lumin of the acinus to be released as SIgA, a complex of the secreted IgA and Secretory component, the cleaved product of pIgR (72).

Flow rate of saliva into the mouth.

Salivary secretion continues throughout the day and the total volume of saliva secreted on average is 500-600 ml. Secretion is low during sleep and very high during eating and drinking. However, the rate at which saliva is delivered to the mouth shows a wide variation between subjects. The properties and composition of mixed saliva in the mouth differ depending upon whether secretion is stimulated by tasting, smelling and chewing food or unstimulated (Table 1). 'Unstimulated' is used to describe a state when no exogenous stimulus is present but in physiological terms there is always some endogenous stimulation in the conscious subject (22). Chewing of inert plastic paraffin film has frequently been used in studies in order to collect larger volumes (approx. 2ml/min) of saliva and under these conditions parotid saliva makes a greater contribution to the saliva in the mouth. A number of studies have examined the flow rate of unstimulated whole mouth saliva (UWMS) and in most of these the mean flow is between 0.3-0.4 ml/ min (85). Dawes et al. found that UWMS flow rate is subject to a circadian variation between a low at approximately 06.00 h and a high at around 18.00 hr (21). It is therefore important to record time of collection when undertaking analyses of UWMS.

Salivary secretion rates differ between males and females and with age. UWMS flow in females is approximately 70% of those in males (23, 43, 85). There is good evidence that UWMS flow rate can decrease in older age. Some researchers have challenged this conclusion based on the link between old age and medication and the link between medication and hyposalivation (see below). However, when non-medicated subjects in old age have been studied the age effect is present (23, 67).

Hyposalivation.

A potential limitation in the use of saliva as a diagnostic fluid is oral dryness due to lack of production of saliva. Dry mouth is most frequently the side effect of the consumption of many prescribed medications and is more prevalent in older age groups; the more medications taken then the more likely it will be experienced. More severe medication induced dryness resulting from salivary hypofunction is associated with anti-cholinergic muscarinic (M3) receptor blockers used to treat, for example, irritable bladders (urinary incontinence). Antidepressants can also cause salivary hypofunction due to activation of alpha-2-adrenergic receptors in the central

nervous system (70). The most severe forms of long lasting, irreversible dry mouth dry mouth are seen in patients with squamous cell carcinoma given external irradiation of the head and neck and sufferers of the autoimmune disease Sjögrens syndrome. In these patient groups the great reduction in saliva can be considered to be a biomarker of salivary gland hypofunction; loss of secretion is due partly to destruction of salivary gland secretory cells but also due to chronic inflammation and the effects of cytokines interrupting normal secretory signaling in salivary glands, a research area requiring further study.

Formation of salivary films and protein pellicles in the mouth.

The volume of saliva in the mouth varies between subjects (0.5-2.1ml) with a mean of 1.1ml just prior to swallowing. Following a swallow the volume is 0.4-1.4ml with a mean of 0.8ml and much of this residual salivary volume is present as a film on all of the mucosal and hard tissue surfaces of the mouth (22). The thickness of the film of saliva has been estimated by measuring the wetness of filter paper strips applied to different surfaces; the anterior tongue has a layer of approximately 50-70 μm, the buccal surface 40-50 μm, whilst the anterior hard palate has a layer of only approximately 10 μm (64). Teeth have a much thinner layer of fluid saliva on the enamel surface. The rate of secretion of saliva into the mouth and the movement of the saliva fluid film over oral surfaces determines how quickly substances are cleared from the mouth through swallowing. This is of great importance to oral health since sucrose, acid and bacterial clearance are required in order to prevent excessive tooth demineralization. Subjects with chronically dry mouth, for example due to the side effects of medications or the salivary gland autoimmune disease Sjögrens syndrome, are particularly susceptible to bacterial or non-bacterial tooth demineralization due to slow salivary clearance (85). In addition to the fluid layer, oral mucoal surfaces have a thin adherent layer of protein, a pellicle (34, 69). The protein pellicle on enamel surfaces was first described some time ago and has been well characterized. In addition to salivary gland derived proteins there are proteins derived from the gingival margins (82, 96). The acquired enamel pellicle provides a lubricating layer that reduces wear and attrition of surfaces and has been shown to reduce acid

induced enamel demineralization providing further protection in addition to that provided by stimulated saliva; thus those tooth surfaces with thicker pellicles are less susceptible to enamel loss compared to those with thin pellicles. Although the enamel pellicle is reduced by tooth brushing and can be almost completely removed by tooth polishing, it rapidly reforms in minutes due to the strongly interacting calcium binding proteins present in saliva (17).

The composition of saliva in the mouth and the effects of reflex stimulation.

Water is actively transported by salivary glands and the flow rate of WMS can increase several fold on stimulation of secretion (see above). In general, the concentrations of salivary components that are actively transported are not greatly reduced in reflexly stimulated saliva; for example amylase, mucins, statherins, proline rich proteins and carbonic anhydrase 6 are maintained at high concentrations in stimulated saliva (63). However, proteins that are not actively transported will tend to decrease in concentration in stimulated compared to unstimulated WMS. For example albumin is present in WMS as a component of crevicular fluid into which it diffuses (89) .

Sodium, chloride and bicarbonate concentrations are increased in stimulated saliva owing to flow rate dependent influences of salivary gland ductal transport of ions (see above). The neutral pH of saliva is maintained by a bicarbonate pH buffering system but the higher concentrations of bicarbonate in stimulated ductal salivas (>20 mM) compared to unstimulated salivas (<5 mM) allows more effective neutralization of dietary acid(2) (10). The reaction of carbon dioxide with water and the bicarbonate buffering reaction are catalysed by carbonic anhydrase 6 and this enzyme may be particularly important in buffering on tooth and mucosal surfaces in the mouth (51). The total calcium concentration of both unstimulated and stimulated WMS is approximately 2 mM which is higher than would be expected for an equivalent water-based solution given the pH of saliva. 'Supersaturation' is achieved by the presence of calcium interacting proteins, most notably statherin, actively secreted by salivary glands (40). Protein bound calcium provides a reservoir for maintaining free calcium levels in saliva. The high calcium and phosphate

concentrations of saliva reduce demineralization of tooth enamel and facilitate subsurface remineralization (80).

Thiocyanate, iodide and nitrate are ions that play an important bacteriostatic role in saliva in conjunction salivary peroxidase in the presence of bacterially derived hydrogen peroxide and this has the effect of limiting bacterial growth at sites of plaque formation (88). Nitrate is reduced to nitrite by bacteria which colonize anaerobic sites on the anterior tongue and nitrite has been shown to limit growth of oral bacteria (59). The generation of nitrite by oral bacteria has recently be shown to also play a role in moderating systemic blood pressure and vasodilation of small vessels through the relaxation of endothelial cells (1, 54).

Physiological considerations in the use of saliva as a biomarker fluid.

UWMS is preferred as a biomarker fluid since the potential variation created by using different types and intensities of reflex stimulation is avoided (see above). One of the disdvantages of using UWMS is that the volume of fluid obtained can be low, particularly in older subjects and those taking xerogenic medications (see above). A broad range of components are present in saliva and represent potential biomarkers of physiology or pathology (Table 2).

The mechanism(s) by which a molecule enters saliva influences the concentrations achieved. Some molecules are lipophilic and can freely cross the salivary and oral epithelium. Saliva is now well established as a biofluid that can provide accurate quantification of circulating levels of free lipophilic steroid hormones (not bound to albumin or other blood proteins) such as cortisol and progesterone which can be repeatedly collected in order establish patterns over time. Although not actively transported many unconjugated steroid hormones are present at similar concentrations in both unstimulated and stimulated saliva. In contrast conjugated steroid hormones such as dihydroepiandrosterone sulphate diffuse through tight junctions between epithelial cells and are present in reduced concentration in stimulated saliva compared to unstimulated saliva (44).

Saliva contains a core proteome made up of relatively abundant proteins actively transported into saliva by glandular epithelial cells (Table 2). These proteins are largely responsible for creating the properties and fulfilling the core functions of

saliva. Proteins from blood enter saliva in relatively small amounts as part of a transudate, crossing tight junctions between epithelial cells which present a diffusion barrier. The concentration of Immunoglobulin G in serum is approximately 11 mg/ml compared to a concentration of only 0.016 mg/ml in saliva (39). The relatively low concentration of IgG in saliva has not prevented detection of specific IgG antibodies directed to viruses, for example HIV or Hepatitis B, as a diagnostic test for infection. This has been achieved by development of collection methods utilizing salt containing absorbant pads which provide an osmotic gradient that increases mucosal transudation and increases capture of tissue fluid derived IgG. This 'saliva' or more accurately 'oral fluid' contains increased IgG levels.

The potential of saliva as a biomarker fluid has been transformed by the development of highly sensitive proteomic analysis which has identified the presence of over 2000 low abundance proteins, approximately 25-30% of which are shared with blood (52)**.** Many of the other low abundance proteins are most likely derived from local oral cellular and tissue sources. A significant source of such proteins is gingival crevicular fluid (GCF) which may be considered a form of tissue transudate. The contribution of GCF is illustrated by the much lower concentrations of salivary albumin in saliva from oedentulous (median = 35 microg./ml) compared to dentate subjects (median = 219 microg./ml) (66, 89). The contributions of GCF to saliva is likely to increase as gingival health deteriorates (66) and it may be that gingivitis and periodontitis can impact on the use of saliva as a biomarker fluid for systemic diseases.

Saliva is an obvious candidate as a biomarker fluid for monitoring oral diseases such as caries, periodontitis and may be also oral cancer since it is a reservoir for products of the affected tissues. Some progress has been made in demonstrating alterations in salivary content of components thought to be involved in oral disease, for example MMP8 (matrix metalloproteinase 8) is a collagen degrading enzyme released by neutrophils and fibroblasts and is elevated in saliva from patients with periodontitis. MMP8 and other proteins elevated in saliva from patients with periodontitis might be used to monitor progression of the disease and response to therapeutic intervention (47). Recent studies indicate that MMP8 is a major collagen degrading enzyme in dentin and its levels also increases in saliva from

patients with caries (42). Antimicrobial peptides, such as LL37 (cathelicidin), alpha-defensins and beta-defensins, derived from neutrophils and epithelial cells can serve as markers of the innate immune response (36).

Exosomes and microparticles are secreted vesicles derived from a variety of different cell types and those isolated from saliva appear to be contributed by salivary and oral epithelial cells and neutrophils (12, 65). Exosomes are 30–100 nm cup-shaped vesicles with a lipid bilayer morphology and unlike other secretory vesicles should contain proteins like CD63 and Alix, which are characteristic of their origin from cellular multivesicular bodies. Salivary exosomes appear to be responsible for the presence of different types of RNA in cell-free saliva including mRNA and various types of non-coding RNA including miRNA, piRNA and circular RNA and there are similarities in the profiles of these molecules in saliva compared to CSF and serum (8). Signals for these molecules are higher in unstimulated compared to stimulated saliva and can be further enhanced by enrichment of exosome/ microparticles from saliva (35).

Conclusion.

Given the ease of collection of whole mouth saliva it is particularly convenient for monitoring physiology and disease. Since the regulation of salivary secretion enables large changes in flow rate and volume of fluid entering and leaving the mouth it is important to carefully standardize saliva collection for biomarker studies. Saliva contains microorganisms and hydrolytic enzymes and so close attention should be paid to the processing and preservation of biomarkers following collection. Use of saliva in assessing systemic levels of steroid hormones and drugs and identifying antibodies to infectious agents has been validated. There is a huge richness of potential salivary biomarkers, including large numbers of proteins, mRNA and non-coding RNA and microbial components with many potential applications. The validation of new biomarkers must also be based on an understanding of how they enter into saliva.

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Figure legends.

1. Ductal system of the submandibular gland demonstrated by sialography. Arrows point respectively to the opening of Wharton's duct sublingually and the branching ductal system of the gland.

2. Histological appearance of salivary gland cells.

The acinar cells of salivary glands have an appearance that depends upon the proteins and glycoproteins stored in granules in the cytoplasm. (A) Acinar cells of parotid glands stain darkly with haematoxylin & eosin (H&E). (B) Minor mucous gland acinar cells contain abundant mucin containing storage granules which are pale staining with H&E. (C) Mucin producing acinar cells of the submandibular gland are stained intensely with alcian blue whilst other non-mucin producing acinar cells are faintly stained with periodic acid Schiff (PAS) reagent. (D) In the sublingual gland acinar cells mostly mucin containing and all are stained with alcian blue. (The images in a-d are taken at different magnifications).

3. Control of salivary secretion by nerves.

The salivary reflex begins with the detection of food and tastants such as acid and salt by tastebuds and mechanoreceptors on the tongue; in addition the chewing of food is detected by mechanoreceptors in the periodontal ligament around teeth. Signals in afferent sensory nerves (green) are relayed to the salivary centres from where efferent parasympathetic nerves (blue) conduct signals to the salivary glands. Sympathetic efferent nerves (red) arise from the thoracic spinal cord. Nerves within the CNS (black) innervate the salivary centres and influence nerve mediated signals to the salivary glands.

4. Intracellular coupling of salivary secretion in acinar cells.

Fluid secretion is dependent mainly upon activation of muscarinic M3 receptors by acetylcholine released from parasympathetic nerves. The intracellular coupling mechanism is characterized by an elevated cytoplasmic calcium released from stores in the endoplasmic reticulum (ER) leading to activation of chloride release.

Protein secretion is mainly activated by release of noradrenaline from sympathetic nerves and activation of beta1 adrenoceptors and vasointestinal peptide released from parasympathetic nerves binding to VPAC receptors. Intracellular signalling is characterized by an increase in cyclic AMP (cAMP) which activates protein kinase A leading to exocytosis of protein storage granules and release of protein into saliva. Gq & Gs - G proteins; PLC – phospholipase C; PIP2 – phosphatidyl inositol biphosphate; IP3 – inositol triphosphate; IP3R – IP3 receptor; AdC – adenylate cyclase; PKA – protein kinase A.

5. Secretion of ionic components of saliva and modification of composition by ducts.

(a) Saliva secretion is dependent upon the low intracellular sodium concentration created by the active sodium pump (ATP). Saliva secretion begins with the movement of sodium and chloride into the acinar lumen; water follows due to the osmotic gradient of salt and enters the acinar lumen by moving between cells or through the water channel, aquaporin 5(Aq5), present in the apical membrane. Different ion transport membranes in the acinar cell membranes allow the salt and water movement: a chloride channel in the apical membrane is opened on glandular stimulation; sodium follows travels between acinar cells through leaky tight junctions (TJL). Chloride enters acinar cells through a chloride co-transporting protein in the basolateral membrane which utilizes the concentration gradient of low intracellular sodium to drive chloride into the cell. Saliva secreted by acinar cells is isotonic. Ductal cells remove sodium and chloride due to the presence of membrane transporter proteins and the low intracellular sodium concentration created by the sodium pump. The tight junctions between ductal cells (TJT) are not leaky and do not allow the movement of water; also there is no water channel in ductal cells. Ductal cells can secrete bicarbonate (HCO_3^-). Following modification by ducts, saliva becomes hypotonic. ATPase – sodium, potassium ATPase; NKCC1 – sodium, potassium, chloride co-transporter; TMEM – TMEM16A calcium activated chloride channel; Na – sodium channel; BCE – bicarbonate, chloride exchanger, Cl – chloride channel; KHE – potassium, proton exchanger.

(b) Immunohistochemical localization of the calcium activated chloride (TMEM16A) in apical membranes of mouse submandibular acinar cells.
(c) Immunohistochemical localization of the sodium, potassium, chloride co-transporter (NKCC1) in basolateral membranes of mouse submandibular acinar cells.

6. Sources of salivary biomarkers.

Salivary fluid is primarily derived from salivary gland secretion. Most of the protein content of saliva is due to salivary proteins synthesized and secreted by salivary acinar cells. However, saliva in the mouth also contains epithelial cells shed from the mucosal surfaces, blood cells (neutrophils) from gingivae and oral microorganisms made up of a rich mix of bacterial species and candida. Small amounts of blood and tissue fluid proteins enter saliva mainly from the gingivae as content of gingival crevicular fluid.

Tables.

Table 1. Rates of flow of whole mouth saliva (WMS) and glandular salivas with and without stimulation.

Footnote for table.

Values given are based on previous studies particularly Pijpe et al., 2007(68)

Table 2. Saliva contains a range of components with potential for diagnostics.

Footnote for table.

Over 2000 proteins have been identified in the salivary proteome including a core group of abundant proteins mainly produced by salivary glands and a large number of low abundance proteins from other sources.

Table 1. Rates of flow of whole mouth saliva (WMS) and glandular salivas with and without stimulation.

	Resting (ml/min)	Resting (%)	Stimulated (ml/min)	Stimulated (%)
WMS	0.35	100	2.0	100
Parotid glands	0.1	28	1.05	53
Submand./ subling. glands	0.24	68	0.92	46
Minor glands	<0.05	4	<0.1	1

Table 2. Examples of the range of different types of salivary component with potential for diagnostics.

Cells and particles:

- Epithelial cells; neutrophils; microorganisms (bacteria (10^8 – 10^9 /ml), viruses, candida, protozoa); Microparticles (0.1-1 µm); exosomes (<0.1 µm)

Proteins and peptide:

Mucin glycoproteins *(MUC5B and MUC7);* Statherin; proline-rich proteins; carbonic anhydrase 6; histatins; secretory component; secretory IgA; IgG; albumin; lysozyme; lactoferrin; MMP8; interleukin 8; nerve growth factor; leptin; LL37; alpha-defensin

Nucleic acid containing molecules:

- DNA; Messenger RNA; non-coding RNA, micro RNA

Steroid hormones:

- Oestrogen, testosterone and cortisol

Lipids

- Triglycerides; cholesterol

Small signaling molecules:

- Adenosine diphosphate

Electrolytes/ ions

- Na^+, Cl^-, Ca^{2+}

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Figure 1

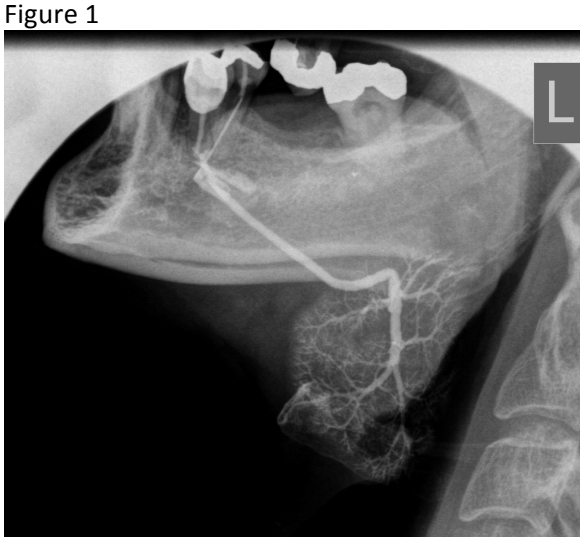

Figure 2

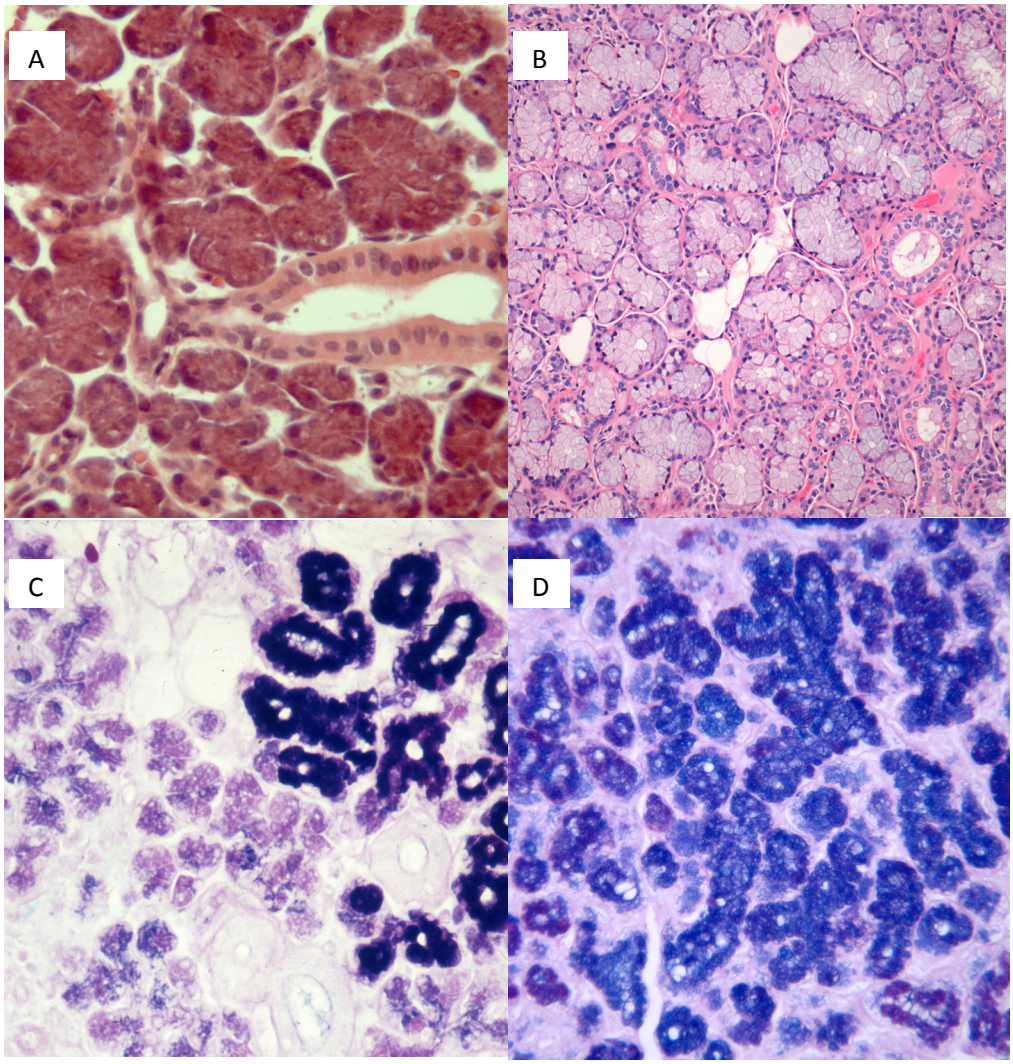

Figure 3

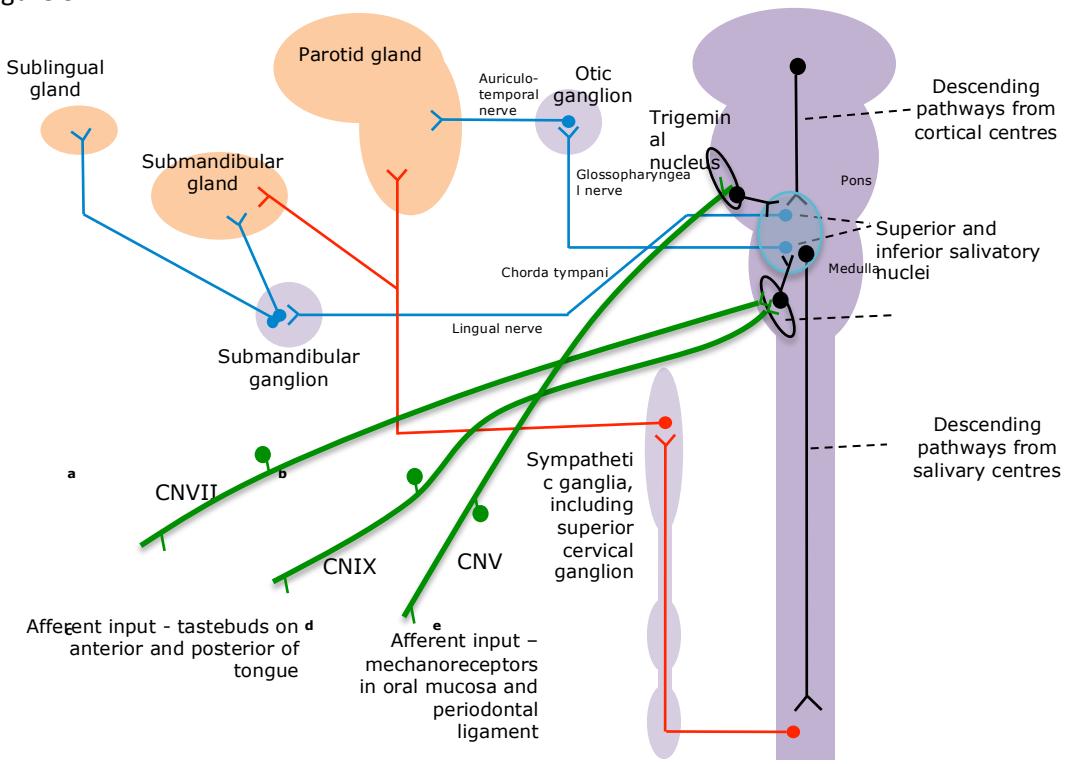
Sublingual gland
Parotid gland
Auriculo-temporal nerve
Otic ganglion
Trigeminal nucleus
Descending pathways from cortical centres
Submandibular gland
Glossopharyngeal nerve
Pons
Superior and inferior salivatory nuclei
Chorda tympani
Medulla
Lingual nerve
Submandibular ganglion
Descending pathways from salivary centres
a
b
CNVII
Sympathetic ganglia, including superior cervical ganglion
CNIX
CNV
c
d
e
Afferent input - tastebuds on anterior and posterior of tongue
Afferent input – mechanoreceptors in oral mucosa and periodontal ligament

Figure 4

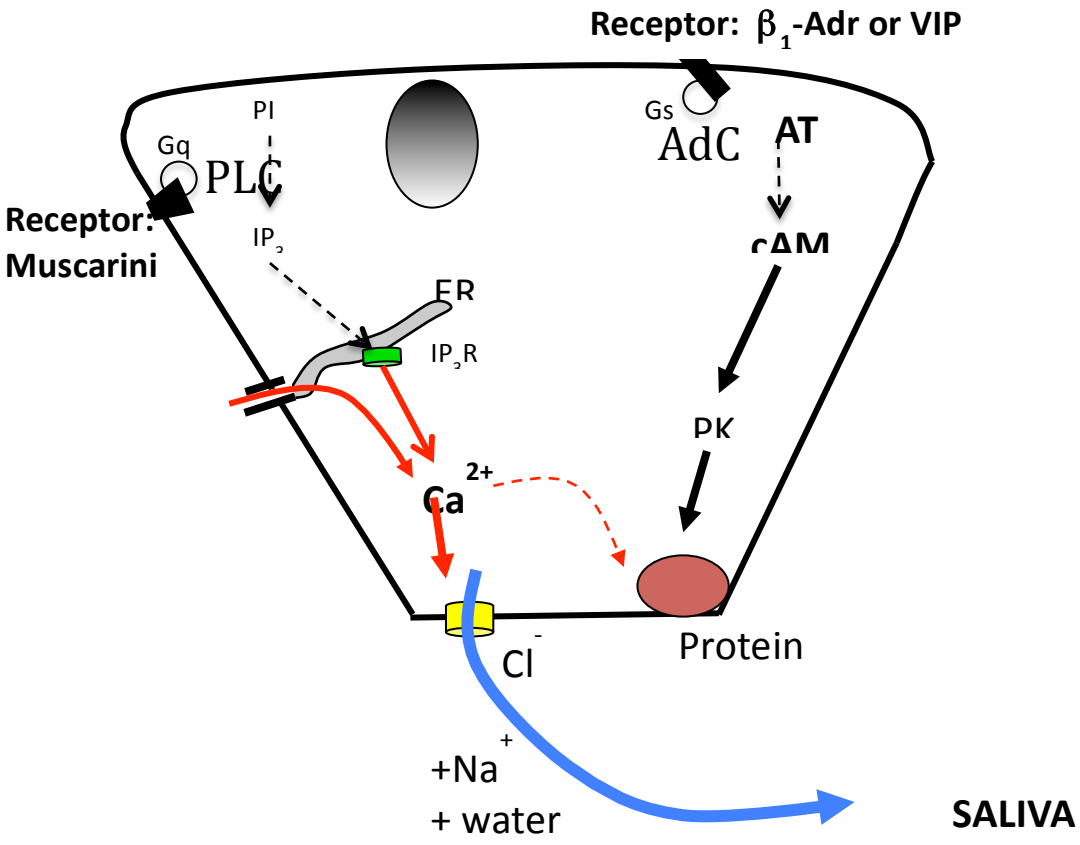
Receptor: β1-Adr or VIP
Gs
AdC
AT
cAM
PK
PI
Gq
PLC
IP3
Receptor:
Muscarini
ER
IP3R
2+
Ca
Cl
-
Protein
+
+Na
+ water
SALIVA

Figure 5a

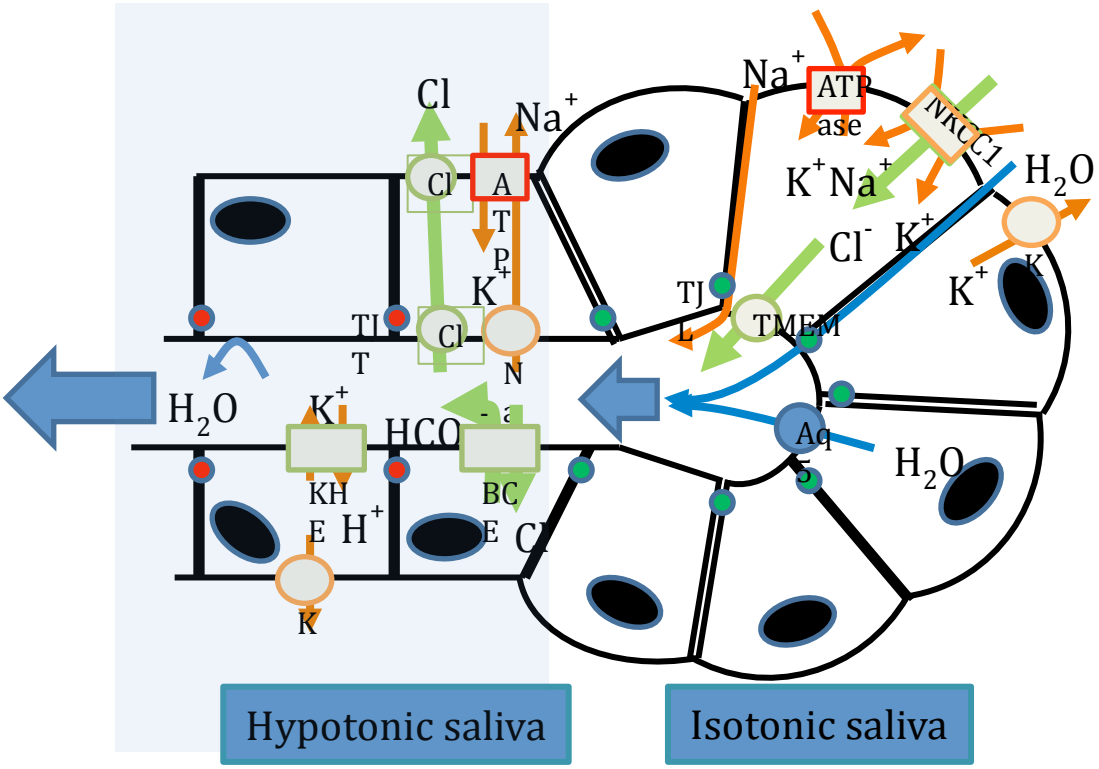
Cl
Na+
Cl
A
T
P
K+
Cl
N
a
TJ
T
H2O
K+
HCO
KH
E
H+
K
BC
E
Cl
Na+
ATP
ase
NKCC1
K+Na+
H2O
Cl- K+
K+
K
TJ
L
TMEM
Aq
5
H2O
Hypotonic saliva
Isotonic saliva

Figure 5b